PB90-265117

Polychlorinated Dibenzo-p-Dioxins and Dibenzofurans Correlation Between In vitro and In vivo Structure-Activity Relationships (SARs)

Texas A and M Univ., College Station

Prepared for:

Environmental Research Lab.-Duluth, MN

1986

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TECHNICAL REPORT DATA
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1. REPORT NO.	2.	3.
EPA/600/J-86/535		PB90-265117

4. TITLE AND SUBTITLE	5. REPORT DATE
Polychlorinated Dibenzo-p-Dioxins and Dibenzofurans: Correlation Between In Vitro and In Vivo Structure Activity Relationships (SARS).	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Safe, S., Mason, G., Keys, B., Farnell, K., Zmudtka, B., Sawyer, T., et al.	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Veterinary Physiology and Pharmacology, Texas A & M University, College Station, Texas 77843.	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO. Grant 810995 RPT 1139
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Duluth, MN 55804	13. TYPE OF REPORT AND PERIOD COVERED
	14. SPONSORING AGENCY CODE EPA-600/03

15. SUPPLEMENTARY NOTES

Published in Chemosphere, Vol. 15, Nos. 9-12, pp. 1725-1731, 1986.

16. ABSTRACT

Polychlorinated dibenzofurans (PCDFs) and dibenzo-p-dioxins (PCDDs) elicit a number of common biologic and toxic responses which are triggered by their initial binding to a cytosolic receptor protein. These effects include the induction of several cytochrome P-448 dependent monooxygenases (eg, aryl hydrocarbon hydroxylase, AHH), body weight loss and thymic atrophy. The dose-response effects of selected PCDFs on AHH induction in rat hepatoma H-4-II E cells and cytosolic receptor binding affinities have been determined. The results of these in vivo and in vitro studies demonstrate the remarkable effects of structure on the activity of PCDFs. A systematic study of each of the four different position for chlorine substitution in the dibenzofuran ring system showed that the toxic and biologic potencies of these compounds varied with respect to differential chlorine substitution at all four position, i.e. C-3(7) > C-2(8) >C-4(6) > C-1(9). In vitro SARs for PCDDs confirmed the importance of the lateral Cl substituents and also showed that 1,2(or 6,7-) substituted PCDDs were more active than the corresponding 1,3-dichloro analogs. In addition, there were significant decreases in activity with increasing non-lateral Cl substitution. The SARs for PCDFs were different from the PCDDs and this was directly related to the asymmetric structure of the former group of compounds.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

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NATIONAL TECHNICAL
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18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to public	Unclassified	7
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

EPA/600/J-86/535

PB90-265117

Chemosphere, Vol.15, Nos.9-12, pp 1725-1731, 1986 0045-6535/86 $3.00 + .00
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POLYCHLORINATED DIBENZO-p-DIOXINS AND DIBENZOFURANS: CORRELATION BETWEEN IN VITRO AND IN VIVO STRUCTURE-ACTIVITY RELATIONSHIPS (SARs)

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ABSTRACT

Polychlorinated dibenzofurans (PCDFs) and dibenzo-p-dioxins (PCDDs) elicit a number of common biologic and toxic responses which are triggered by their initial binding to a cytosolic receptor protein. These effects include the induction of several cytochrome P-448 dependent monooxygenases (eg, aryl hydrocarbon hydroxylase, AHH), body weight loss and thymic atrophy. The dose-response effects of selected PCDFs on AHH induction in rat hepatoma H-4-II E cells and cytosolic receptor binding affinities have been determined. The results of these in vivo and in vitro studies demonstrate the remarkable effects of structure on the activity of PCDFs. A systematic study of each of the four different position for chlorine substitution in the dibenzofuran ring system showed that the toxic and biologic potencies of these compounds varied with respect to differential chlorine substitution at all four position, i.e. C-3(7) > C-2(8) >C-4(6) > C-1(9). In vitro SARs for PCDDs confirmed the importance of the lateral Cl substituents and also showed that 1,2(or 6,7-) substituted PCDDs were more active than the corresponding 1,3-dichloro analogs. In addition, there were significant decreases in activity with increasing non-lateral Cl substitution. The SARs for PCDFs were different from the PCDDs and this was directly related to the asymmetric structure of the former group of compounds.

INTRODUCTION

Polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs) and related halogenated aryl hydrocarbons elicit a number of common toxic and biologic effects in animal species and mammalian cells in culture (1-7). These effects include body weight loss, thymic atrophy and or immunotoxicity, endocrine disorders, gastric lesions, hepatoxicity, chloracne and other dermal lesions, teratogenicity and reproductive problems and carcinogenicity. The more toxic halogenated aryl hydrocarbons, typefied by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), induce a diverse spectrum of enzymes including the cytochrome P-448 dependent monooxygenases benzo[a]pyrene (aryl hydrocarbon) hydroxylase (AHH) and ethoxyresorufin O-deethylase (EROD). The induction of these enzyme activities and the associated cytochrome P-450 isozyme, cytochrome P-450c, is one of the most sensitive and characteristic responses of exposure to 2,3,7,8-TCDD and related isostereomers. The development of the characteristic toxic responses to these compounds are species-dependent: however, two of these effects, namely body weight loss and thymic atrophy (or immunotoxicity), are observed in most animal species.

This paper, summarizes and compares the in vivo and in vitro quantitative SARs for PCDDs and PCDFs which have been developed in our laboratory using AHH/EROD induction (in vivo and

in vitro), body weight loss (in vivo) and thymic atrophy (in vivo) as end points (8-11). ... hepatoma H-4-II E cells in culture were used to determine EC_{50} values for enzyme induct... ED_{50} values for *in vivo* enzyme induction and toxicity were measured using immature m... Wistar rats as the animal model; competitive receptor binding affinities were also determi... using male Wistar rat hepatic cytosol.

MATERIALS AND METHODS

All the PCDF and PCDD congeners were synthesized as described (9-11) and were > 9...% pure.

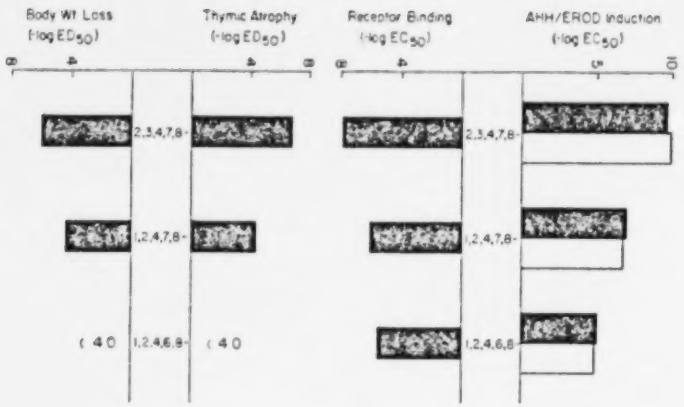
The *in vitro* induction of AHH and ethoxyresorufin O-deethylase (EROD) in rat hepatoma ... 4-II E cells were determined as described (8-11). The ED_{50} values were defined as t... concentration of chemical which effects 50% of the maximal enzyme induction observed in t... dose-response procedure used for each compound. The relative binding affinities for th... cytosolic receptor protein were determined by competitive displacement of [^{3}H]-2,3,7,8-TCD... The EC_{50} values for each congener were defined as the concentrations required to displac... one-half the radioligand from the receptor protein.

The *in vivo* enzyme induction and toxicologic studies were carried out as described (10). The PCDDs or PCDFs in corn oil were administered by intraperitoneal injection to male Wistar rats and the body weights were determined every second day. The animals were killed thirteen days after the first exposure to the toxin and the thymuses were excised, blotted and weighed. Hepatic microsomal AHH and EROD activities were determined as described (9-11).

RESULTS AND DISCUSSION

Polychlorinated Dibenzofurans

The SARs for PCDFs as inducers of AHH and EROD in rat hepatoma H-4-II E cells and as competitive ligands for the rat hepatic cytosolic receptor protein were comparable. The most active compounds were substituted in the lateral 2,3,7, and 8 positions and a decrease in the number of lateral substituents resulted in decreased induction potencies and receptor binding affinities (see Table 1). Comparable SARs were observed for the *in vivo* enzyme induction and toxic potencies for the PCDFs and this is illustrated in Figure 1. for a series of pentachlorodibenzofurans (PeCDFs) which contain 4 (2,3,4,7,8-PeCDF), 3 (1,2,4,7,8-PeCDF) and 2 (1,2,4,6,8-PeCDF) lateral Cl substituents. It was also evident (Figure 2) that for several series of PCDF isomers which differed only in substitution at C-4(or C-6) and C-1 (or C-9) (eg. the 1,2,3,7,8-/2,3,4,7,8-, 1,2,3,7,9-/2,3,4,7,9-, 1,2,3,7-/ 2,3,4,7-isomers the C-4 (or C-6) isomer pairs were more active. Figure 2 illustrates this effect by comparing the *in vitro* and *in vivo* activities of the 2,3,4,7,8- and 1,2,3,7,8-PeCDFs. A comparison of the relative activities of 1,3,4,7,8- and 1,2,4,7,8- PeCDF and 2,3,4,7 and 2,3,4,8-TCDF showed that isomers substituted at C-3 (or C-7) were more active than the C-2 (or C-8) chloro-substituted analogs (see Figure 2). Based on ED_{50} values for body weight loss and thymic atrophy, 2,3,4,7-TCDF was 4-16 times more toxic than 2,3,4,8-TCDF and 1,3,4,7,8-PeCDF was 1.8-65 times more toxic than 1,2,4,7,8-PeCDF. The results clearly illustrate that all four positions in the dibenzofuran ring contribute differentially to the activity of PCDFs [i.e.



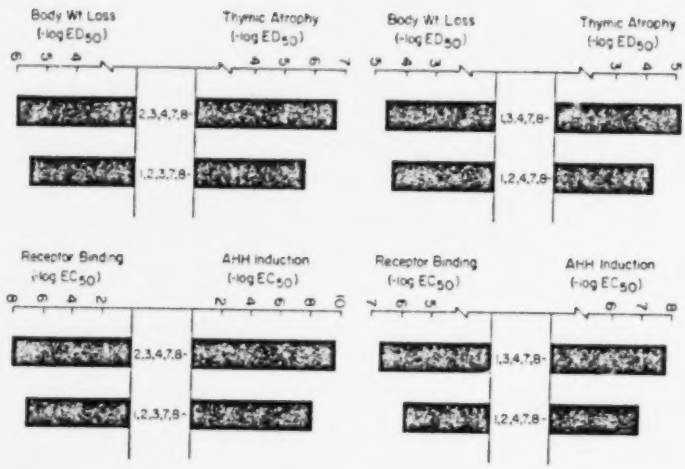


Figure 1 (Top). Effects of structure on the activities of 2,3,4,7,8-, 1,2,4,7,8- and 1,2,4,6,8-PCDF. Figure 2 (bottom). Effects of structure on the activities of 2,3,4,7,8-, 1,2,3,7,8-, 1,3,4,7,8- and 1,2,4,7,8-PCDF.

C-3 (or C-7) > C-2 (or C-8) > C-4(or C-6) > C-1 (or C-9)].

Polychlorinated Dibenzo-p-dioxins

We have recently completed (11) an extensive study on the in vivo and in vitro SARs for PCDDs and not surprisingly, 2,3,7,8-TCDD was the most active compound. It was evident that the induction activities, receptor binding avidities and toxicities of a series of 2,3,7,8-substituted PCDD congeners (eg 1,2,3,7,8-, 1,2,3,4,7,8- and 1,2,3,4,6,7,8,9-) decreased with increasing non-lateral Cl substituents. In addition there were other important substitution effects which influence receptor binding avidities. The only difference in structure between the 1,3,7,8- and 2,3,6,7-TCDD isomers is the presence of a 1,3 or 1,2 (or 6,7)-dichloro substitution pattern, however the five-fold difference in their competitive binding EC$_{50}$ values clearly illustrates that interactions with the receptor protein are more favored by ligands with the latter substitution pattern. Similar SARs have been noted for isosteric PCDFs (9,10). It was also evident that there was a decrease in activity with decreasing lateral Cl substitution. The hepatic microsomal AHH and EROD inducing activities and toxicities (body weight loss and thymic atrophy) of 2,3,7-trichloro-, 2,3,7,8- and 1,3,7,8-tetrachloro-, 1,2,4,7,8- and 1,2,3,7,8-pentachloro- and 1,2,3,4,7,8-hexachlorodibenzo-p-dioxin were determined in a dose-response fashion in immature male Wistar rats. It was evident that the in vivo SARs for this limited number of PCDD congeners were similar to the results obtained using the in vitro enzyme induction and receptor binding assays (see Figure 3).

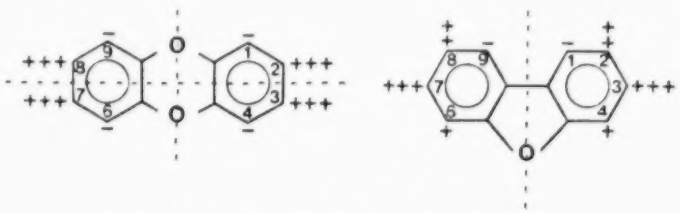
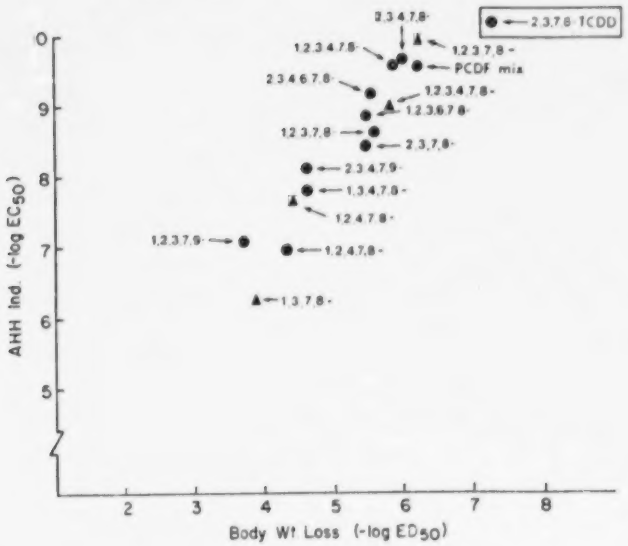


Figure 3. PCDDs and PCDFs - Summary of SARs

QSARs for PCDFs and PCDDs:

In Vivo and In Vitro Correlations Our studies have demonstrated that for PCDD and PCDF congeners comparable qualitative SARs were observed using the in vitro receptor binding and enzyme induction assays (AHH and EROD) and the in vivo responses (hepatic microsomal AHH and EROD induction; thymic atrophy and body weight loss) in immature male Wistar rats. Moreover the data from several other studies which utilized a limited number of congeners and other animal species also support the proposed SARs for this class of compounds (1-7). These results suggest that it is practical to develop and validate specific short term in vitro bioassays which can quantitatively predict the potential toxicity of a sample containing PCDDs, PCDFs and related toxic halogenated aryl hydrocarbons (9-14). We have previously proposed that both the in vitro receptor binding and enzyme induction bioassays could be utilized for this purpose (15). However, it was apparent that for PCDD and PCDF congeners there was not a linear correlation between their receptor binding avidities and AHH/EROD induction potencies (9-11). It was apparent for eleven PCDF congeners that there was a



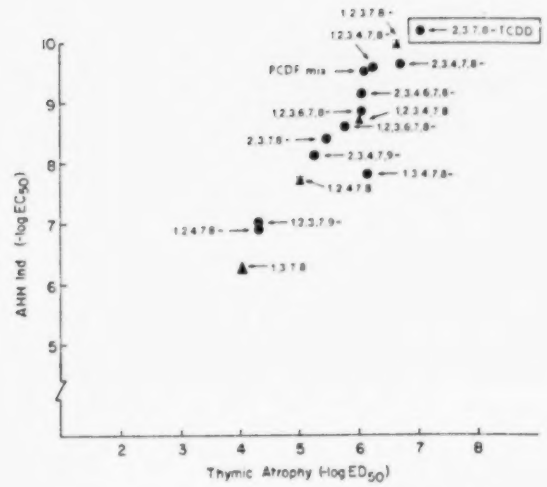


Figure 4 (top). A plot of -log EC_{50} (in vitro AHH induction) vs -log ED_{50} body weight loss (rat). Figure 5 (bottom). A plot of -log EC_{50} (in vitro AHH induction) vs -log ED_{50} thymic atrophy (rat).

excellent linear correlation between a plot of the -log ED_{50} for body weight loss vs. -log EC_{50} for *in vitro* AHH induction (Figure 4) (correlation coefficient, r=0.96) and -log ED_{50} for thymic atrophy vs. -log EC_{50} for *in vitro* AHH induction (correlation coefficient, r=0.88) (Figure 5). The data summarized in Figures 3 and 4 for five of the six PCDD congeners also illustrates the linear relationship between the -log EC_{50} values for enzyme induction and the -log ED_{50} data for two of the receptor-mediated *in vivo* responses. The *in vivo* results obtained for 2,3,7-trichlorodibenzo-p-dioxin did not correlate with the *in vitro* AHH induction data and this is consistent with the expected rapid metabolism of this compound. Comparable results were noted for PCDF congeners which contained two adjacent unsubstituted positions disposing them towards rapid metabolism (9,10).

If the dose-response data for the five PCDD congeners reported in this study and the eleven PCDFs (including one reconstituted mixture) (9,10) are analyzed together, the linear correlation coefficients for a plot of -log EC_{50} (in vitro AHH induction) vs - log ED_{50} (body weight loss) and -log ED_{50} (thymic atrophy) are 0.823 and 0.827 respectively. Since body weight loss and thymic atrophy in the rat are representative toxic responses to toxic halogenated aryl hydrocarbons, the correlations noted above support the use of the *in vitro* AHH induction assay as a short term quantitative test system for this class of toxic halogenated aryl hydrocarbons.

Further studies with other animal species using different toxic responses are required to validate the applicability of these induction assays as a quantitative screening method for toxic halogenated aryl hydrocarbons. Moreover, the assay must also be tested using reconstituted and environmental mixtures of PCDFs and PCDDs; many of these validation studies are currently in progress in our laboratory.

ACKNOWLEDGEMENTS

This research was supported by U.S. Environmental Protection Agency, the Texas Agricultural Experiment Station and Natural Sciences and Engineering Research Council of Canada.

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ACC: PB90-264904

03A) ANN: GRA [ ] VGA [ ] DB [ ]

03G) GRAPHICS: [ ]

30A) [2] 0 N/A 4 FOR 1 Am-I 5 For-T 2 R 6 For-R 3 T

025) SUBJECT CATEGORIES: 68D, 57Y

23D) DESCRIPTORS

* Toxicology,
* Aquatic biology,
Temperature,
Lipids,
Oxygen consumption,
Aromatic polycyclic hydrocarbons,
Bioaccumulation,
Fish,
Toxicity,

25I) IDENTIFIERS

* Pharmacokinetics,
* vertebrates,
* Hexagonal lambda,
* Water pollution effects(Animals),
Environmental monitoring,
Polychlorobiphenyl compounds,
Passive biological transport,
Cell membrane,

27A) ABSTRACT

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1. REPORT NO.	2.	3.
EPA/600/J-88/531		PB90-264904

4. TITLE AND SUBTITLE	5. REPORT DATE
Toxicokinetics of Selected Xenobiotics in Hexagenia Limbata	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Landrum, P.F., Poore, R.	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Nat'l Oceanic and Atmospheric Admin., Great Lakes Environmental Research Lab., 2205 Commonwealth Blvd., Ann Arbor, MI 48105	10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO. CR DW13931213-01-01
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Duluth, MN 55804	13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/03

15. SUPPLEMENTARY NOTES
Published in: J. Great Lakes Res.: 14(4):427-437, 1988

16. ABSTRACT

ABSTRACT. *Understanding the role of benthos in the fate and transport of contaminants requires understanding the toxicokinetics of those organisms for both waterborne and sediment-associated compounds. This effort focused on the toxicokinetics of* Hexagenia limbata *as an important component of the benthic community of the upper Great Lakes connecting channels. The accumulation and elimination of two polycyclic aromatic hydrocarbon congeners and a hexachlorobiphenyl isomer were followed over the course of a season in* H. limbata *collected from Lake St. Clair. Both the water uptake clearance and elimination rate constants increased with increasing temperature through the spring and summer. The elimination constants were relatively large. The uptake constant for sediment-associated compounds was essentially constant the two times it was measured and was large compared to sediment accumulation by* Pontoporeia hoyi. *Steady state model calculations indicate that the amount of compound in* H. limbata *should decline as temperature increases. The BCF values on a lipid content normalized basis remain relatively constant for both PAHs and show some change with season for hexachlorobiphenyl. Based on the best estimates of environmental concentration of the contaminants studied in both sediment and water, the model suggests that* H. limbata *should obtain greater than 90% of its contaminant body burden from the sediment-associated pollutants.*
ADDITIONAL INDEX WORDS: Toxic substances, mayflies, benthos, path of pollutants.

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18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to public	Unclassified	11
	20. SECURITY CLASS (This page)	22. PRICE
	Unclassified	

PB90-264904

J. Great Lakes Res. 14(4):427–437
Internat. Assoc. Great Lakes Res., 1988

EPA/600/J-88/531

TOXICOKINETICS OF SELECTED XENOBIOTICS IN *HEXAGENIA LIMBATA*

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ABSTRACT. *Understanding the role of benthos in the fate and transport of contaminants requires understanding the toxicokinetics of those organisms for both waterborne and sediment-associated compounds. This effort focused on the toxicokinetics of* Hexagenia limbata *as an important component of the benthic community of the upper Great Lakes connecting channels. The accumulation and elimination of two polycyclic aromatic hydrocarbon congeners and a hexachlorobiphenyl isomer were followed over the course of a season in* H. limbata *collected from Lake St. Clair. Both the water uptake clearance and elimination rate constants increased with increasing temperature through the spring and summer. The elimination constants were relatively large. The uptake constant for sediment-associated compounds was essentially constant the two times it was measured and was large compared to sediment accumulation by* Pontoporeia hoyi. *Steady state model calculations indicate that the amount of compound in* H. limbata *should decline as temperature increases. The BCF values on a lipid content normalized basis remain relatively constant for both PAHs and show some change with season for hexachlorobiphenyl. Based on the best estimates of environmental concentration of the contaminants studied in both sediment and water, the model suggests that* H. limbata *should obtain greater than 90% of its contaminant body burden from the sediment-associated pollutants.*
ADDITIONAL INDEX WORDS: Toxic substances, mayflies, benthos, path of pollutants.

INTRODUCTION

Hexagenia limbata are an important food source for fish in the interconnecting waterways of the Great Lakes (Hunt 1958). Further, *H. limbata* are extremely sensitive to pollution, resulting in their disappearance from some locations in bays and the connecting channels (Carr and Hiltunen 1965, Howmiller and Beeton 1971, Schneider *et al.* 1969, Hiltunen and Schloesser 1983). While the disappearance has been attributed to low dissolved oxygen as a result of eutrophication, oil inputs into the St. Marys River below Sault Ste. Marie, Michigan, have eliminated the mayfly larvae from stretches of the river that they previously inhabited (Hiltunen and Schloesser 1983). The mayfly larvae are beginning to repopulate areas of the Great Lakes (Thornley 1985). Since *H. limbata* live and feed in the sediment, the mayfly larvae may mobilize contaminants from the sediments up through the food chain. Because of their sensitivity to pollution and their importance in the food web, we undertook to define the toxicokinetics of these organisms for two polycyclic aromatic hydrocarbons (PAH) and one polychlorinated biphenyl (PCB) congener. The studies were run over the course of a field season to determine the seasonal variability that occurs in the toxicokinetics resulting from environmental variables such as temperature and physiological variables such as changes in lipid content.

MATERIALS AND METHODS

H. limbata were collected seven times over the course of a field season beginning in early May 1986 and continuing until November 1986 on approximately monthly intervals. The collection site was near the middle of Lake St. Clair, at Loran coordinates 49934 and 31128. The depth was approximately 20 ft. The animals were collected by PONAR grab, gently screened from the sediment, and placed in a container of lake water and sediment for transport back to the laboratory (generally about 2 h). The bottom temperature was taken at the same time the animals were collected. The

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animals were maintained in the laboratory in aerated (50 L) aquaria containing approximately 3 cm lake sediment and 10 cm lake water at the temperature of collection.

The compounds studied were ^{3}H-benzo(a)pyrene (BaP)(specific activity 23.8 Ci/mmol, Amersham), ^{14}C-phenanthrene (Phe)(specific activity 14 mCi/mmol, Pathfinder Laboratories) and ^{14}C-2,4,5,2',4',5'-hexachlorobiphenyl (HCB)(Specific activity 14.06 mCi/mmol, Pathfinder Laboratories). All compounds were determined to be at least 98% radiopure prior to use by thin layer chromatography (TLC) and liquid scintillation counting (LSC). The purity check was performed on silica gel plates (E Merck, 250 μm) using hexane:benzene (8:2 V:V) as the solvent system. The plate was scraped at the same Rf as a standard and several sections lower. These scrapings were placed in scintillation vials and counted. The counts below the Rf of the standard were assumed to be degradation products. All analytical procedures were performed under gold fluorescent lights ($\lambda > 500$ nm) to minimize the degradation of the PAH congeners.

Toxicokinetic studies were performed within 1 week of the time of collection. The accumulation from water was performed in quadruplicate as a flow-through experiment. The infusion rate into the 200-mL exposure chambers was 100 mL h^{-1} of dosed water. The temperature for each experiment was the same as the environmental temperature measured at the time of the collection (Table 1). Water, collected from Lake St. Clair at the same time as the animals, was filtered prior to use through glass fiber filters (Gelman AE). The water was dosed in bulk (5 L) with the radiolabeled compounds using a methanol carrier and allowed to equlibrate for 1 h prior to starting the flow and introducing the organisms. The concentration of the methanol as a carrier was < 50 mg L^{-1} and was not expected to influence the kinetics (Landrum 1983). Because there was no sediment substratum in the water-only exposures, the animals were provided with artificial burrows to minimize potential thigmotactic stress (Henry *et al.* 1986). The artificial burrows used in these studies were made from stainless steel screen instead of glass tubes. Preliminary studies with glass tubes indicated a depletion of compound concentration within the tubes, resulting in a very large variation in the accumulation of compound by the organisms (P. F. Landrum, unpublished data). One or two animals were removed from each of the four replicates after 1, 2, 4, and 6 h exposure, blotted dry, weighed, and placed in scintillation cocktail for radioanalysis. The time course for the accumulation experiments was based on the response of other Great Lakes invertebrates for the accumulation of organic contaminants from water (Frez and Landrum 1986, Frank *et al.* 1986, Landrum *et al.* 1985a). In addition to the animal concentrations, the water concentration of the contaminant was determined in each exposure chamber at each time point by placing 2 mL of water directly into scintillation cocktail for radioanalysis. The amount of compound in the water and organisms was based on the amount of radioactivity and the specific activity of the compound under study. Binding of the study compound to dissolved organic matter (DOM), that can reduce the biological availability of non-polar organics in water (Landrum *et al.* 1987, 1985b), was measured at the midpoint of the uptake experiment by passing an aliquot of water through a C-18 Sep Pak® and measuring the amount of DOM-bound contaminant passing through the column (Landrum *et al.* 1984). The uptake clearance was corrected for the fraction of compound bound to DOM.

The organisms remaining in the exposure chamber after the uptake phase had been completed were removed and placed in uncontaminated sediment for elimination. Elimination rates were determined by removing one or two animals from each of two to four replicates at approximately 1, 3, 5, 7, and 14 d, rinsed of sediment, blotted dry, weighed, and placed at scintillation cocktail for radioanalysis. Actual times were used for kinetic determinations.

The uptake clearance constants were determined by fitting the accumulation data to a constant infusion model using initial rate assumptions. The initial rates assumptions for the model require that the water concentration not change over the course of the uptake phase and that elimination is unimportant. The data can then be fit to the following equation:

$$C_a = K_u C_w t \qquad (1)$$

where C_a is the concentration in the organism (ng g^{-1}), K_u is the uptake clearance constant (mL g^{-1} h^{-1}), C_w is the concentration in the water (ng mL^{-1}) and t is time (h). The concentration in the water had the following coefficients of variation: HCB 11%, BaP 9%, Phe 6.3%. The uptake was measured over a period that is less than 15% of one

TABLE 1. Oxygen consumption and lipid content for **Hexagenia** *in 1986.*

Date	Oxygen Consumption $\mu g\ O_2\ mg^{-1}\ h^{-1}$ (mean ± S.D.)	Oxygen Clearance[1] $mL\ g^{-1}\ h^{-1}$	%Lipid	Temperature °C
May	0.327 ± 0.111 n = 5	23.3 ± 7.9	7.8 ± 1.9 n = 7	10
June	Lost		15.1 ± 2.6 n = 5	15
July	0.667 ± 0.296 n = 5	41.4 ± 18.3	9.1 ± 3.4 n = 8 4.3 ± 1.8[2]	15
August	0.435 ± 0.10 n = 5	61.9 ± 13.5	3.6 ± 1.0 n = 7	20
September	0.25 ± 0.06 n = 10	44.5 ± 12	6.0 ± 2.4 n = 6 6.9 ± 1.9[2]	20
October[3]	0.16 ± 0.10 n = 4	18.6 ± 11.8	3.7 ± 1.2 n = 7 3.3 ± 0.9[2]	20
November	ND		6.0 ± 1.4 n = 4	10

[1]The n for the clearance determination is the same as the oxygen consumption determination.
[2]Samples collected in 1985.
[3]Sample actually collected on 30 September 1986, oxygen consumption was run 60 d after collection.
ND - not determined

elimination half-life even for the fastest elimination rate for BaP and HCB but in some cases nearly 60% of one elimination half-life for some of the Phe data. Plots of the data appeared linear except for the Phe data for May 1986 that dropped off at the last time point. The linear portion of the curve was used for calculation of the uptake clearance. The r^2 values for the combined data sets were often low (approximately 0.2 to 0.4), especially for HCB, resulting in large variance in the uptake clearance estimates. The variation resulted primarily from large differences between replicates and occasional spurious data points. Individual replicates generally had r^2 values in the range of 0.65 to 0.98. Because of the linear appearance of the data and the generally small fraction of the elimination half life that occurred and the relatively small coefficients of variation for the water concentrations, the model was presumed to be appropriate and we believe that the assumptions were not violated.

The elimination data were fit to a simple first order decay model of the form:

$$C_s = C_s^{\circ}\, e^{-K_d t} \qquad (2)$$

where C_s and t have the same definition as above and K_d is the elimination rate constant (h^{-1}) and C_s° is the initial concentration in the organism at the beginning of the elimination experiment.

The accumulation from sediment was measured by sorbing the radiolabeled compounds onto sediments in an aqueous slurry overnight. The sediment was transferred to a large beaker and allowed to settle at 4°C for 24 h. The overlying water was decanted and the sediment mixed and distributed to 18 beakers (approximately 75 g per beaker). The actual weights of the sediment in each beaker were determined. Subsamples of the sediment were taken for analysis of the concentration of radiolabeled compound and for wet to dry weight of the sediment. The beakers, six per aquaria, were placed in three 6-L aquaria, and approximately 3 L of overlying Lake St. Clair water added to cover the beakers with approximately 5 cm of water. The aquaria were allowed to stand for an additional 24 h before adding the organisms. One *H. limbata* was added per beaker and a screen was placed over the top of the beaker until the organism had burrowed into the sediment. The mayfly nymphs burrowed almost immediately. Three organisms were removed for uptake, one from each of three

aquaria, at approximately 1, 3, 5, 7, and 14 d (actual times were used for determination of kinetics). The animals were rinsed of sediment, blotted dry, weighed, and placed in scintillation cocktail for radioanalysis. Samples of sediment were taken at each sample point for analysis of contaminant concentration and for wet to dry weight of sediment. All sediment concentrations were based on the dry weight of sediment. Overlying water was taken and analyzed as above to ensure that the concentration of the radiolabeled compound was at background.

Accumulation data were fit to a two compartment model with a source function that could show exponential decay to account for changes in the concentration of the contaminant in the sediment and for potential changes in the biological availability of the contaminant:

$$dC_a/dt = K_s C_s^{\circ} e^{-\lambda t} - K_d C_a \qquad (3)$$

where:

C_a = Concentration in the organism (ng g^{-1})
C_s° = Initial concentration in the sediment (ng g^{-1})
K_s = Uptake clearance from sediment (g sediment g^{-1} animal h^{-1})
λ = Rate constant for reduction in the bioactivity of the contaminant in the sediment (h^{-1})
K_d = elimination rate constant (h^{-1})
t = time (h)

The partitioning of organic contaminants into reversible and resistant pools on particles results in reductions in the extractability of organic contaminants (DiToro *et al.* 1982, Karickhoff and Morris 1985). This separation into these two pools is relatively slow compared to the initial association with particles. A similar two-phase separation into biologically available and biologically unavailable contaminant was found for *Pontoporeia hoyi* accumulation of contaminants from sediment, and this effect was greatest for the more water soluble compounds such as Phe (P. F. Landrum, unpublished data). Because the sediments were prepared for exposure in a relatively short time period and because the time required for partitioning into the two pools may be relatively long, the λ term was included in the accumulation equation. For sediments that have had time to achieve steady state, probably most natural sediments, the λ value would not be required in an accumulation model. The uptake clearance from sediment and λ, the rate of change of the bioavailable source, were estimated from the data fit by nonlinear regression using PROC NLIN (SAS 1985) and supplying the K_d value measured in the elimination experiment.

Sediment concentrations were determined by extraction of the sediment after desiccation with anhydrous sodium sulfate. The sediments were stored under 100 mL ethylacetate at room temperature until analysis. Initial extraction methodology was by Soxhlet extraction of the sediment in ethylacetate and cyclohexane 50:50 overnight. However, equivalent results were obtained by allowing the samples to sit for 2 to 3 weeks after mixing with the ethylacetate at the time of sampling and separating the solvent by filtration. Subsequent soxhlet extraction after filtration did not yield additional removal of radiolabeled material. Concentrations of the contaminants were determined by radioanalysis of a known fraction of the extracting solvent. The bulk of the extract was reduced in volume by a combination of rotary evaporation and evaporation under a stream of nitrogen to a final volume of approximately 500 μL. Then a portion of the extract was chromatographed by TLC using hexane:benzene 8:2. The extent of degradation was determined as described above for the pre-use purity check.

Organisms exposed through water were analyzed for biodegradation products by extraction in ethylacetate:acetone 4:1 (2 × 20 mL) followed by extraction with cyclohexane (1 × 20 mL). The extraction was accomplished by macerating the animals in a tissue grinder with the extracting solvent. The extracting solvents were combined and filtered from the residue of the carcass. The solvent was reduced in volume and the biotransformation determined as was done for the sediment extracts. The carcass residue was analyzed for radioactivity as described for whole animals. The extent of metabolism was determined as the amount of non-parent compound found by TLC and the amount activity remaining in the residue.

Radiometric analyses were performed on an LKB 1217 scintillation counter using the external standards ratio method. Additional measures made over the course of the season included lipid content and weight measurements. The lipid content was determined with a microgravimetric method (Gardner *et al.* 1985a). Wet weight measures were made within a few days after collection. These animals were desiccated to a constant dry weight and subsequently ashed at 500°C for 1 hour and the ash weight determined.

Oxygen consumption was determined at the

same temperature as the kinetics experiments and performed within 1 week of collection, except for the 30 September collection where the oxygen consumption was run 60 d after collection. One animal was placed in each of five to ten 300-mL BOD bottles, the bottles capped, and the oxygen concentration determined after removing the animal. The duration of the experiment was 24 h. The concentration in the water was compared to a control containing the same water but no organisms. The oxygen consumption normalized to biomass was determined by the Winkler titration using 0.005 M thiosulfate (Grasshoff *et al.* 1976). Clearance constants for oxygen, equivalent to the uptake clearance constants of the contaminants, were calculated from oxygen consumption and the concentration of oxygen in the control bottles.

A model of toxicokinetics for *H. limbata* was employed to predict steady state body burdens for comparison with other organisms and with available field data. The model used the general differential equation:

$$dC_a/dt = K_w C_w + K_s C_s - K_d C_a \quad (4)$$

where dC_a/dt is the change in the organism concentration per unit time; K_w is the uptake clearance constant from water (mL g^{-1} h^{-1}); C_w is the concentration of contaminant in water (ng mL^{-1}); and the remainder of the terms are as defined above. The λ term was dropped from the model because the sediments in natural systems would presumably be at steady state with respect to the partitioning between reversible and resistant pools. The rate constants employed for the model were those determined in the laboratory studies. The steady state solutions for Eq. 4 are:

$$C_{ss} = (K_w C_w + K_s C_s)/K_d \quad (5)$$

$$C_a^w = K_w C_w / K_d \quad (6)$$

$$C_a^s = K_s C_s / K_d \quad (7)$$

where C_{ss} is the concentration of the contaminant in the organism from both a water and sediment source at steady state, C_a^w is the concentration in the animal at steady state from water (ng g^{-1}), and C_a^s is the concentration in the animal at steady state from sediment (ng g^{-1}). From these equations the fraction from water can be computed from C_a^w/C_{ss}; similarly the fraction from sediment can be computed from C_a^s/C_{ss}.

RESULTS

The dry to wet weight ratio and ash free dry to dry weight ratio determinations were made three times over the course of the season. The dry to wet weight ratio was 0.18 ± 0.02 (mean ± sd, n = 3), while the ash free dry to dry weight ratio was 0.75 ± 0.10 (n = 3). Each determination employed 5 to 10 organisms. The lipid content was low and appeared to peak at the beginning of June (Table 1) and ranged from about 5 to 6% of dry weight from about August through the fall (Table 1).

Oxygen consumption also appeared to peak in the summer and declined toward the fall (Table 1). Respiration rates in the spring and fall were significantly lower than rates in the summer while respiration rates in the fall were significantly lower than spring ($p < 0.05$, Student's t test). The increase in oxygen consumption in the summer, over those measurements in the spring and fall, was probably due in part to increases in temperature. However, the low oxygen consumption in October was at the highest environmental temperature examined. This low October value was probably due in part to the long hold time in the laboratory before the measure was made (approximately 60 d) and should not necessarily be considered representative for the time of year. If the low value is incorrect then the oxygen consumption increases with increasing temperature as expected.

The toxicokinetics for accumulation from water were measured on a monthly basis over the course of the field season for 1986 (Table 2). The uptake of contaminants from water generally seemed to rise in the summer and decline again in the fall for all the compounds. The rise in uptake clearance for the HCB seemed to lag that of the PAH. The uptake clearances for HCB generally had larger variances than those for the PAH. The reasons for this are not clear.

The elimination rate constants also showed the same trend of an increase in the summer and a decrease in the fall. These changes generally tracked changes in the temperature at which the experiments were performed. Bioconcentration factors (BCF), calculated from the kinetics in water, showed a general trend of higher BCF in the spring and summer for BaP while the BCF for HCB and Phe were essentially constant over the course of the season (Fig. 1). No biotransformation was attributable to the organisms, although some overall degradation was observed for BaP. Between 4 and 5% of the total BaP radioactivity

TABLE 2. Seasonal uptake clearance and elimination rate constants for **Hexagenia limbata.**

Month	Benzo(h)pyrene	Phenanthrene (mean ± S.D.)	Hexachlorobiphenyl	Temp.
May Ku*	68.5 ± 11.2	131.1 ± 46.8	47.5 ± 23.9	10[1]
Kd**	0.011 ± 0.003	0.032 ± 0.004	0.007 ± 0.001	
June Ku	67.0 ± 28.0	43.3 ± 12.0	44.2 ± 8.0	15
Kd	0.006 ± 0.002	0.0076 ± 0.0016	0.005 ± 0.002	
Ks***	0.043 ± 0.005	0.065 ± 0.016	0.030 ± 0.01	
	0.025 ± 0.004[2]			
July Ku	101.9 ± 32.6	57.5 ± 5.0	40.8 ± 37.3	15
Kd	0.013 ± 0.002	0.029 ± 0.002	0.005 ± 0.001	
Aug Ku	65.1 ± 29.1	11.9 ± 4.0	40.8 ± 37.3	20
Kd	lost	lost	0.007 ± 0.001	
Ks	0.035 ± 0.005	0.042 ± 0.008	0.09 ± 0.02	
Sept Ku	149.5 ± 29.0	56.3 ± 6.8	128.7 ± 20.3	20
Kd	0.016 ± 0.003	0.032 ± 0.004	0.015 ± 0.003	
Sept[3] Ku	76.3 ± 41.0	33.0 ± 8.0	95.0 ± 17.3	20
Kd	0.028 ± 0.001	0.067 ± 0.008	0.017 ± 0.002	
Nov Ku	40.9 ± 30.6	34.2 ± 7.2	45.5 ± 16.1	10
Kd	0.010 ± 0.001	0.026 ± 0.002	0.004 ± 0.0006	

*Ku has been corrected for sorption to dissolved organic carbon and has units of mL $g^{-1}h^{-1}$.
**Kd has units of h^{-1}.
***Ks has units of g dry sediment g^{-1} animal h^{-1}.
1. Temperature is in degrees centigrade.
2. Uptake from sediment was measurement twice for BaP.
3. This collection was actually made on 30 September 1986.

was found to be non-parent. The degradation did not increase with time as is expected for PAH undergoing biotransformation and has been observed with other organisms (Leversee *et al.* 1982). The degradation was not observed for Phe or HCB.

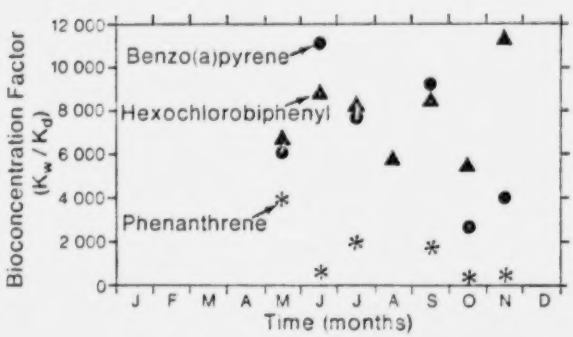

FIG. 1. Calculated bioconcentration factors in **Hexagenia limbata** *over the 1986 field season calculated from the ratio of* K_u/K_d.

The sediments used in the studies had an organic carbon content of 6.98%. The size fractionation of the sediment indicated that 31.9% of the sediment mass was < 74 μm in diameter. This fine-grained material had an organic carbon content of 8.3%. The determination of the uptake clearance from sediment required the use of a model that could account for changes in both the chemical and bioavailable concentration of the contaminant in the sediment. The extractable concentration of the contaminants often showed some declines with time over the course of the studies: BaP 0 to 11%, Phe 0 to 33%, and HCB 0 to 58%. These changes plus the changes in biological availability are accounted for with the λ value in the kinetics model. The λ values found for the data were in the range of 0 to 0.007 h^{-1}, with the larger λ values paralleling the larger changes in chemical availability. The accumulation from sediments was essentially constant the two times it was measured

(Table 2), but was measured only twice due to the time required for performance of the experiments and the analysis of the samples generated.

DISCUSSION

The accumulation of neutral organic xenobiotics from water is assumed to occur as passive diffusion across the respiratory membrane of the aquatic organism. As expected, the uptake clearance increased with increasing temperature, indicating increased activity. The variances of all the accumulation kinetics constants were generally greater than had been previously observed for other Great Lakes invertebrates (Frez and Landrum 1986, Frank *et al.* 1986). This variance may have been due in part to the stress of not having a substrate to burrow into even though we supplied artificial burrows for the animals to relieve this stress. This is supported in part by a somewhat smaller variation in the accumulation from a sediment matrix compared to the water-only exposure (Table 2). Another possible reason for the increased variance would be differential "pumping" of the gills between different animals. Some of the *H. limbata* seemed to have a regular rhythm while others appeared to not move their gills at all. This would result in a differential volume of water passing across the animal and thus a differential uptake. It is not possible to determine whether either or both of the above reasons account for the relatively large variance observed in the uptake clearance.

The uptake clearances appeared to be relatively independent of the octanol-water partition coefficient (K_{ow}) of the compounds. However, the clearance measured for Phe was generally lower than that measured for BaP, two of the same class of compounds. On average the uptake clearances were about the same size or lower compared to other aquatic invertebrates (Table 3). At equivalent temperatures, the accumulation clearances for BaP from water for *H. limbata* were lower than those for *Mysis relicta*, a Great Lakes invertebrate of similar size (Frez and Landrum 1986). Further, the uptake clearance for *H. limbata* was smaller for BaP than that of *Chironomus riparius* run at similar temperatures (Leversee *et al.* 1982) although *C. riparius* is a smaller organism and uptake clearances seem to be inversely related to size.

Oxygen consumption measurements for *H. limbata* were similar to those found in the literature for *H. limbata* (Zimmerman *et al.* 1975). In general the increased clearance of oxygen paralleled the increased clearance of the organic contaminants from water. From these measurements, the uptake clearance for oxygen was determined (Table 1) for comparison with the uptake clearance constants for the contaminants (Table 2). In most cases, the contaminant was cleared from the water with a greater efficiency than the oxygen based on the higher clearances for the contaminants compared to oxygen. This increased efficiency suggests that the integument as well as the respiratory membrane is a route for some of the uptake.

The thermal regime also influences the elimination rate resulting in increased K_d values with increased temperature. The elimination rate constants were inversely related to K_{ow}, with the K_d for Phe > BaP > HCB. The elimination rate constants are large and likely result in part from the relatively low lipid content of *H. limbata*. The lipid content of *H. limbata* is low compared to other Great Lakes invertebrates and was most comparable with the oligochaetes (Gardner *et al.* 1985b). The chironomid larvae, another insect larvae in the Great Lakes, contain nearly twice the lipid content of *H. limbata* (Gardner *et al.* 1985b). The elimination rate constants were about equivalent to those of *M. relicta* and were larger than those of *Pontoporeia hoyi* (Table 3). *C. riparius*, another insect larvae, have a larger apparent elimination rate constant for BaP but the *C. riparius* have a much better biotransformation capability (Leversee *et al.* 1982) than *H. limbata* (This work).

If one uses the ratio of K_a over K_d to calculate a bioconcentration factor (BCF)(Fig. 1), the BCF appears to remain fairly constant over the sampling season for HCB and Phe but BaP shows some decline with season, indicating that the K_d value increased relative to K_a. *H. limbata*'s calculated BCFs were of similar size to the other Great Lakes species except *P. hoyi* (Table 3). The high *P. hoyi* BCF occurs because of the absence of measureable biotransformation (Landrum 1988) and the high lipid content of the organism (Gardner *et al.* 1985b). Normalization of the calculated BCF to the fraction of lipid (concentration of contaminant in the lipid divided by concentration in the water) results in annual average normalized BCFs of 29,427 ± 13,964 for Phe, 89,578 ± 32,982 for BaP, and 125,818 ± 47,677 for HCB (Table 4). The log of the normalized BCF is inversely related to the log of aqueous solubility by log BCF = 3.44 ± 0.20 - 0.192 ± 0.028 log S (r^2 = 0.74), where S is the contaminant aqueous solu-

TABLE 3. Comparison of the toxicokinetics for **Hexagenia limbata** *with other Great Lakes invertebrates.*

Organism/Rate Constant	Average Kinetics Constants PHE	BaP	HCB
Hexagenia limbata Temperature range 10–20 °C[1]			
K_w	52.5	81.3	63.2
K_d	0.032	0.014	0.009
K_s	0.054	0.034	0.06
BCF(K_w/K_d)	1,640	5,870	7,022
Pontoporeia hoyi 4°C			
K_w[2]	129.0	116.8	NA
K_d[2]	0.0046	0.0016	NA
K_s[3]	0.041	0.0029	0.0057
BCF(K_w/K_d)	28,043	40,275	NA
Stylodrilius heringianus 4°C[4]			
K_w	94.0	87.8	NA
K_d	0.016	0.012	NA
K_s	0.159	0.0011	NA
BCF(K_w/K_d)	5,875	7,316	
Mysis relicta 4°C[5]			
K_w	32.0	112.0	NA
K_d	0.012	0.013	NA
K_s	NA	NA	NA
BCF(K_w/K_d)	2,667	7,466	NA

1. This work
2. Landrum 1988
3. P. F. Landrum, unpublished data
4. Frank *et al.* 1986
5. Frez and Landrum 1986

K_w = uptake clearance from water, K_s = uptake clearance from sediment, K_d = elimination rate constant, and BCF = bioconcentration factor

TABLE 4. Lipid-normalized calculated bioconcentration factors for **Hexagenia limbata.**

Month	Benzo(a)pyrene	Phenanthrene	2,2′4,4′5,5′-hexachlorobiphenyl
May	79,836	52,484	86,996
June	73,951	37,731	58,543
July	86,137	21,788	89,670
August	ND	ND	161,904
September	155,729	29,323	143,000
October	73,648	13,312	151,033
November	68,166	21,923	189,583

ND = Not determined because the elimination rate constant was not available.

bility in mol L^{-1} from Buggeman *et al.* (1982). With the lipid normalization, the BaP and Phe normalized BCF values are relatively constant throughout the measured period (Table 4). HCB on the other hand has BCF values that are approximately double during the months of August through November compared to May through June (Table 4). The HCB data suggest that the lipid content alone may not be sufficient to predict the BCF but that lipid composition may play a role.

Accumulation from sediments was larger than has been observed for *P. hoyi* for the PAH studied (Landrum *et al.* 1985a, P. F. Landrum, unpublished data). However, comparing *H. limbata* with sediment accumulation by *Stylodrilius heringianus*, the oligochaetes accumulated BaP more slowly than the *H. limbata* but accumulated Phe more rapidly (Frank *et al.* 1986). Part of the differences between the various organisms and the rates of accumulation from sediments include the temperature, sediment composition, and species differences. *H. limbata* was examined at 15 and 20°C while *P. hoyi* and *S. heringianus* were studied at 4°C. The sediment organic carbon content for the *H. limbata* was 6.8% while for the other two organisms the sediment contained 1% for *P. hoyi* (P. F. Landrum, unpublished data) and 5% for *S. heringianus* (Frank *et al.* 1986). In addition, the accumulation by *S. heringianus* was performed with sediments pre-seived to 250 μm (Frank *et al.* 1986). Thus, from a high organic carbon content whole sediment, *Hexagenia* have relatively high rates of accumulation.

The assimilation efficiency from sediments can be estimated from literature values of feeding rates (Zimmerman and Wissing 1978) and the K_s values. Our organisms were generally 20 mm in length or longer and K_s was determined at 15 and 20°C. The feeding rates for the larger nymphs were 0.21 g sediment g^{-1} organism h^{-1} at 15°C and 0.31 g g^{-1} h^{-1} at 20°C after converting the feeding rates based on dry weight nymphs to a wet weight basis using our dry weight ratio and converting to from daily to hourly averages (Zimmerman and Wissing 1978). These feeding rates have the same units as the K_s values; thus, it is possible to compare the feeding rates directly with the K_s values. Since the K_s values are less than the feeding rate, the implication is that all of the contaminant is not being removed from the sediment as it passes through the gut of the organism. Therefore a ratio of K_s to feeding rate should give the fraction of material assimilated or removed. With these feeding rates the efficiency for assimilation for BaP ranged from 11.3–3.21%, Phe from 13.6–13.3%, and HCB from 14.4–29.1%. The estimated assimilation efficiency for HCB is in the same range as that determined for oligochaetes in an elegant dual-labeled study (Klump *et al.* 1988).

Using a steady state approach and comparing the results of equations 6 and 7, the relative accumulation from the water as a source can be compared to that of sediment. Literature values of concentrations of the contaminants in water and sediment were used to reflect the environment from which the animals were taken. The concentrations of HCB were 45 ng g^{-1} in sediment and 0.0032 ng g^{-1} in water (Smith *et al.* 1985). The estimate for the water concentration of HCB was calculated as the total PCB concentration in water times the fraction of the PCB mixture represented by HCB as measured in the sediment (0.07) where congener analysis had been done. The BaP and Phe concentrations in water were 0.012 and 0.024 ng g^{-1} respectively (Eadie *et al.* 1983) and in the sediment were 425 and 325 ng g^{-1} respectively as determined in Lake Erie sediments (Eadie *et al.* 1982). Using the steady state model for the two times that the HCB accumulation from sediment was measured, the fraction of the body burden from the water was estimated to be 0.1 and 0.03. Thus the fraction from sediment was 0.9 and 0.97. The main route of HCB accumulation for this organism is apparently via the sediment. Similar comparisons for the PAH yielded ratios for BaP of 0.9 and 0.96 and for Phe 0.95 for both determinations as the fraction of contaminant obtained from the sediments. This determination is very dependent on the ratio of the products of K_wC_w and K_sC_s; therefore, changes in environmental concentrations without any change in rate constants would alter the fraction obtained from a particular source.

Comparing the estimated role of sediment as a source with other organisms, *H. limbata* apparently obtains a greater fraction of its body burden from the sediment. Using the same water and sediment concentrations as for the projections for *H. limbata* and the kinetics constants for the oligochaete, *S. heringianus*, (Frank *et al.* 1986) and *P. hoyi* (Landrum *et al.* 1985a), the oligochaetes would obtain 34 to 67% of their BaP body burden from sediment while *P. hoyi* would only obtain 39% from sediments. This suggests that the role of sediments as a source will depend on the organism as well as the sediment characteristics.

The steady state approach can be used to calculate a bioaccumulation factor (BAF) (organism concentration/sediment concentration) by using equation 5 and dividing the result by the sediment concentration. The steady state concentration was divided by the sediment concentration because it was presumably the predominant source for the organisms. The λ term from the general sediment accumulation model can presumably be ignored because the sediment materials are assumed to be

at equilibrium with respect to the resistant and reversible pools of compound. How true this assumption is in reality is impossible to tell since sorption time is unknown in real systems and sediment presumably contains a mixture of particles with various times of sorption. The BAF ranged from about 4.5 to 15.5 for HCB and reflects the generally low elimination rate constant while PAH showed a much lower BAF, Phe 0.9 - 2.2 and BaP 1.5 - 3.8. The BAF predicted for *H. limbata* are higher than those found for oligochaetes for HCB in the field (Smith *et al.* 1985) and BaP (Eadie *et al.* 1982) but were about the same as oligochaetes for Phe (Eadie *et al.* 1982). Comparing *H. limbata* to *P. hoyi*, the range of BAFs is about the same for the two PAHs studied (Eadie *et al.* 1985). One set of unpublished data permits direct comparison the BAF for HCB in *H. limbata*. The BAF for *H. limbata*, collected from a site in Lakes St. Clair close to the one used for these studies, was 0.54 for a late July collection based on a sediment concentration of 1.76 $\pm$ 0.15 $\mu g\ kg^{-1}$ and a *H. limbata* concentration of 0.94 $\pm$ 0.22 $\mu g\ kg^{-1}$ (D. C. Bedard, Dept. of Biological Sciences, Univ. of Windsor, Windsor, Ontario; Personnal Communication, 1985). No water value was available for calculation using equation 5. However, if the sediments account for 90–97% of the body burden then equation 7 should provide a reasonable estimate of the steady state body burden. From equation 7, the predicted concentration in *H. limbata* would be 10.5 - 22.6 $\mu g\ kg^{-1}$ with the resultant BAF of 6.0 to 12.8. The difference between the predicted BAF and the measured value is about an order of magnitude. The reasons for these differences may be several. First, the organisms may not ingest sediment material but fresh detrital material that may have a lower HCB concentration; this would result in a lower BAF. Second, the animals in the natural environment may not be at steady state due to changing thermal conditions. Certainly more efforts are required to obtain more comparable data, and these should be available with the publication of the studies of the Upper Great Lakes Connecting Channels project supported by the U.S. Environmental Protection Agency.

In conclusion, *H. limbata* appears to obtain the preponderance of its body burden of the contaminants examined from the sediment, based on calculations from the toxicokinetics and sediment and water data from the literature. The accumulation from water seems to occur both through the gills and the body wall, based on the relative clearances of oxygen and contaminants. The accumulation from sediment seems to occur with about the same assimilation efficiency as was observed for oligochaetes. The BCF may be dependent on not only the total lipid content but on the composition of the lipid based on the doubling of the BCF for HCB during the latter half of the study. The predicted BAF seems to be high compared to the measured values and more effort will be required to determine the reasons.

ACKNOWLEDGMENTS

This work was jointly supported by the Great Lakes Environmental Research Laboratory, NOAA, and by the U.S. Environmental Protection Agency through interagency agreement No. DW13931213-01-01. I wish to thank Tom Fontaine for his critical discussions and suggestions on the kinetics modeling. I also wish to thank Brian Eadie, Tom Nalepa, Mike Quigley, and Guy Stehly for their critical review of this manuscript. GLERL Contribution Number 570.

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